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Reassessment of *Clostridium difficile* Susceptibility to Metronidazole and Vancomycin

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***Clostridium difficile* is the most frequently identified enteric pathogen in patients with nosocomially acquired, antibiotic-associated diarrhea. The drugs most commonly used to treat diseases associated with *C. difficile* are metronidazole and vancomycin. Most clinical laboratories assume that all *C. difficile* isolates are susceptible to metronidazole and vancomycin. We report on the antimicrobial susceptibilities of 415 *C. difficile* isolates to metronidazole and vancomycin over an 8-year period (1993 to 2000). The overall rate of resistance to metronidazole at the critical breakpoint (16 μg/ml) was 6.3%. Although full resistance to vancomycin was not observed, the overall rate of intermediate resistance was 3.1%. One isolate had a combination of resistance to metronidazole and intermediate resistance to vancomycin. Rates of resistance to metronidazole and vancomycin were higher among isolates from human immunodeficiency virus-infected patients. Molecular typing methods proved the absence of clonality among the isolates with decreased susceptibilities to the antimicrobials tested.**

Clostridium difficile-associated diarrhea (CDAD) is the most common nosocomial diarrhea in adults, occurring mainly in patients with prior antimicrobial therapy. The disease has variable incidences and severities in different hospital populations (2, 6, 22, 25, 27, 38). Recent information also points to CDAD as a relatively frequent cause of community-acquired episodes of diarrhea [24; T. V. Riley, M. Cooper, B. Bell, and C. L. Golledge, abstract from the 5th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 1995, Clin. Infect. Dis. **20**(Suppl. 2)**:**S263–S265, 1995].

The most serious cases of CDAD require antimicrobial therapy with agents which are active against *C. difficile*. Metronidazole is the drug of choice due to its in vitro activity, its efficacy by either the oral or the intravenous route of administration, its presumed lower potential for selection of vancomycin-resistant *Enterococcus* (VRE), and the low cost of treatment with the drug. Nowadays, vancomycin is considered a second-line drug, mainly due to the potential for the selection of VRE and its high cost. Both drugs are considered equivalent in efficacy, but infection recurrence rates of 15 to 35% have been reported for both drugs (3, 7).

In vitro determination of the susceptibility of *C. difficile* to these antibiotics is not routinely performed, as it is broadly accepted that *C. difficile* is regularly and predictably susceptible to metronidazole and vancomycin. The assay method is time-consuming, and the use of susceptibility breakpoints is based on the therapeutic levels of drugs in serum and not on the levels in the intraluminal area, where higher drug concentrations can be achieved (1, 19). At present, metronidazole resistance in *C. difficile* is considered anecdotal and vancomycin resistance has been, to the best of our knowledge, reported only once (16), although on that occasion the method used was not the present standard.

In 1994, we sounded the alarm when we reported on the first series of metronidazole-resistant *C. difficile* isolates (T. Peláez, R. Sánchez, R. Blázquez, P. Catalán, P. Muñoz, and E. Bouza, Abstr. 34th. Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-34, p. 50, 1994). The aim of the present study is to report on the decrease in the rates of susceptibility to metronidazole and vancomycin in some of the *C. difficile* isolates in our institution over an 8-year period (1993 to 2000).

MATERIALS AND METHODS

Patients and strains. During the study period we identified 2,384 toxigenic strains of *C. difficile* from 1,787 patients. Of these, 415 isolates from 415 hospitalized adults with their first CDAD episode were randomly selected for study.

C. difficile isolates were presumptively identified by their colony morphology, yellow color, ground-glass texture, characteristic horse-dung smell, and Gram staining and fluorescent properties. Additional biochemical tests were also used (32).

The demonstration of toxin production was carried out either directly from fecal samples or, if the result was negative, from a "second-look" assay with strains isolated on cycloserine-cefoxitin-fructose agar medium (12, 44). The presence of *C. difficile* toxin B was determined by demonstrating a specific cytopathic effect on MRC-5 cells, as described previously (32, 41).

MIC determination by agar dilution. MICs were determined by the agar dilution method described by the National Committee for Clinical Laboratory Standards (35). All assays were performed in duplicate.

The antimicrobial agents used in the study, metronidazole and vancomycin, were obtained from Sigma (St. Louis, Mo.). Serial twofold dilutions of the antimicrobials were incorporated into supplemented brucella agar (Oxoid, Basingstoke, United Kingdom), with antibiotic concentrations ranging from 0.016 to 32 μg/ml. Inocula were prepared from supplemented brucella broth (Oxoid), in which the organisms were grown at 37°C for 24 h. Cultures were adjusted to an optical density on the McFarland scale of 0.5, and 10 μl (10^5 CFU/spot) was applied with a Steers replicator to prereduced brucella agar. The plates were observed after 48 h of incubation in an anaerobic chamber (85% nitrogen, 10% hydrogen, 5% carbon dioxide) at 37°C.

The MIC was defined as the lowest concentration of the agent that inhibited growth. The appearance of one or two colonies or a barely visible haze was disregarded. Reference strains (*Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741) were included as controls to monitor the antimicrobial susceptibility testing. A collection strain of *C. difficile* (ATCC 9689)

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TABLE 1. Chronological distribution of nonsusceptible *C. difficile* isolates

Agent	No. (%) of nonsusceptible isolates in:								
	1993	1994	1995	1996	1997	1998	1999	2000	Total
Metronidazole	3 (6.2)	6 (9.6)	6 (12.5)	3 (6.4)	2 (2.8)	3 (4.6)	2 (4.1)	1 (4)	26 (6.3)
Vancomycin	0 (0)	0 (0)	0 (0)	2 (4.2)	2 (2.8)	6 (9.2)	2 (4.1)	1 (4)	13 (3.1)

was also included to assess the reproducibilities of the assays. The breakpoints for metronidazole were ≤8 μg/ml for susceptible, 16 μg/ml for intermediate, and ≥ 32 μg/ml for resistant. For vancomycin we used breakpoints of ≤2 μg/ml for susceptible, 4 to 16 μg/ml for intermediate, and ≥32 μg/ml for resistant, as no standard is defined by the NCCLS.

Bacterial typing. A subset of 10 isolates which were representative of those obtained from different areas of the hospital throughout the study period from patients belonging to different risk groups and which had phenotypes of decreased susceptibility to both study drugs (metronidazole and vancomycin) were studied for clonal relatedness. Random amplification of polymorphic DNA (RAPD) analysis was performed as described previously (3). Oligonucleotides T7 (5′-GTAATACGACTCACTATAG-3′) (46) and RAPD-#3 (5′-GTAGACC CGT-3′; Amersham Pharmacia Biotech, Uppsala, Sweden) were used to prime the RAPD reactions.

Statistical methods. The significance of differences in susceptibility patterns was analyzed by the chi-square or the two-tailed Fisher exact test. A P value of <0.05 was considered statistically significant.

RESULTS

The incidence of CDAD per 1,000 admissions at our institution increased from 2.1 in 1993 to 8.9 in 2000. The most common underlying diseases of our patients were as follows: 294 were human immunodeficiency virus (HIV)-positive patients (16.5%), 139 were organ transplant patients (7.8%), 106 were oncology patients (5.9%), and 1,248 had a miscellany of other diseases (69.8%)

Susceptibility to metronidazole. The metronidazole MIC at which 50% of isolates tested are inhibited (MIC_{50}) and the MIC_{90} for the 415 strains assayed were 0.5 and 4 μg/ml, respectively (range, 0.016 to >32 μg/ml).

A total of 26 strains of *C. difficile* (6.3% of all strains tested) were resistant to metronidazole. The distribution of resistant strains throughout the study period is shown in Table 1. The rate of resistance ranged from 2.8 to 12.5%. The distribution of the MICs for the nonsusceptible strains is shown in Table 2.

The rate of resistance was not uniform among the groups with different underlying conditions (Table 3) and was significantly higher among the isolates from HIV-infected patients (12.6%) than among the isolates from the other patient groups ($P < 0.001$).

Susceptibility to vancomycin. The vancomycin MIC_{50} and MIC_{90} for the 415 assayed strains were 1 and 2 μg/ml, respectively (range, 0.016 to 16 μg/ml).

Strains not susceptible to vancomycin were first detected in our laboratory in 1996, although none of our isolates showed full resistance to this agent. The evolution of vancomycin-intermediate isolates throughout the study period is summarized in Table 1. Overall, 3.1% of all our isolates of *C. difficile* were not susceptible to vancomycin. The distribution of the MICs for the nonsusceptible strains is shown in Table 2.

The distribution among the different groups of patients of strains not susceptible to vancomycin is shown in Table 3. The proportions ranged from 0 to 4.2%, although the differences were not statistically significant.

A high degree of genetic diversity was detected among the isolates studied by typing analysis (Fig. 1).

TABLE 2. Distribution of metronidazole and vancomycin MICs for nonsusceptible *C. difficile* isolates

Agent	No. of isolates for which the MIC (μg/ml) is:				
	4	8	16	32	>32
Metronidazole			0	3	23
Vancomycin	0	4	9	0	0

DISCUSSION

C. difficile is the most frequently identified causal agent of hospital-acquired diarrhea. Advanced age, severe underlying disease, recent surgery, and, much more importantly, the prior use of antimicrobial therapy are among the predisposing conditions related to CDAD (10, 17, 28, 29, 33, 45, 48). Populations at higher risk also include those undergoing organ transplantation and HIV-infected patients [3, 5, 6, 11, 26, 30, 51; P. Muñoz, T. Peláez, J. Palomo, R. Muñoz, M. J. Ruiz Serrano, and E. Bouza, abstract from the 7th ECCMID 1997, Clin. Microbiol. Infect. **3**(Suppl. 2)**:**218, 1997].

The basis for adequate therapy relies on the rapid diagnosis of either toxin A or toxin B, or both, directly from fecal samples or from strains of *C. difficile* isolates obtained from feces by a negative direct test (a "second-look" assay for cytotoxicity) (3, 4, 12, 13, 15).

Metronidazole is the drug of choice for the treatment of first episodes of CDAD. It is effective when it is administered by either the oral or the parenteral route, has a low potential for selection of VRE, and is inexpensive. Nowadays, vancomycin is considered a second-line drug, mainly due to its potential for the selection of VRE, its efficacy only with oral administration, and its high cost. Both drugs are considered to have equivalent efficacies, and both are associated with infection recurrence rates of 15 to 35% (7–9, 14, 18, 19, 21, 31, 39, 47, 50).

C. difficile is considered susceptible to metronidazole in most institutions, and tests to assess the in vitro activity of metronidazole against *C. difficile* isolates are rarely performed in most

TABLE 3. Number of isolates of *C. difficile* and percent resistance to metronidazole and vancomycin, by patient risk group

Agent	No. (%) of isolates from:			
	HIV-infected patients (n = 95)	Transplant recipients (n = 38)	Oncology patients (n = 15)	Other patients (n = 276)
Metronidazole	12 (12.6)	1 (2.6)	1 (6.6)	12 (4.3)
Vancomycin	4 (4.2)	1 (2.6)	0 (0)	8 (2.9)

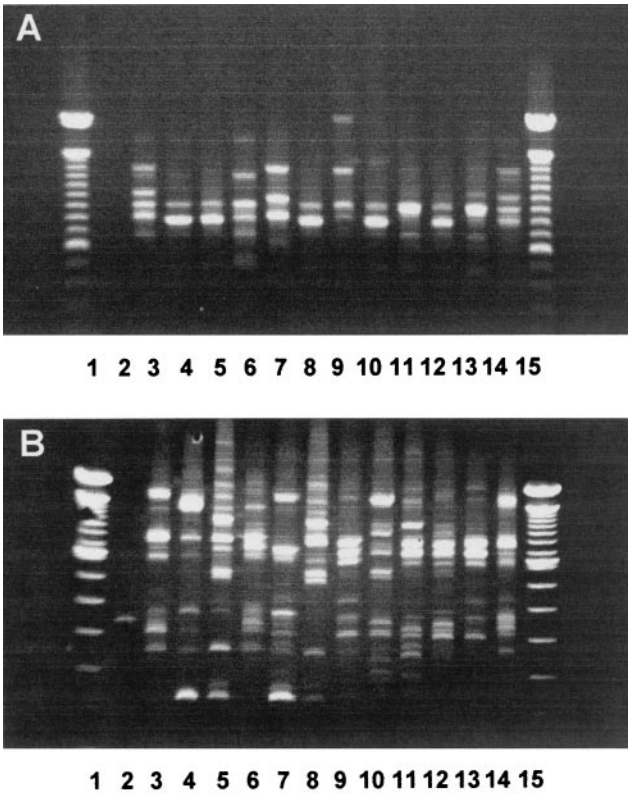

FIG. 1. RAPD typing patterns of a representative subset of isolates. (A) Primer T7 (low level of discrimination); (B) primer RAPD-#3 (high level of discrimination). Lanes 1 and 15, molecular weight markers; lane 2, negative control; lanes 3 and 14, collection strain (*C. difficile* ATCC 9689); lanes 4 to 13, clinical *C. difficile* isolates.

centers (20, 23, 34, 36, 50). It is well known, however, that CDAD may develop in patients during metronidazole therapy (43) and that isolates of *C. difficile* resistant to metronidazole in vitro had already been reported anecdotally in 1981 (37), although these facts are not necessarily related.

In 1994, our group reported a 6% rate of resistance to metronidazole among 78 isolates of *C. difficile* (Peláez et al., 38th ICAAC). In another study (T. Peláez, L. Alcalá, L. Martínez-Sánchez, P. Muñoz, J. M. García-Lechuz, M. Rodríguez-Créixems, and E. Bouza, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-173, p. 219, 1998), we found that 9% of strains were resistant (MICs, $\geq$32 μg/ml). Other recent reports from different investigators have also warned about this problem. In 1997, high-level metronidazole resistance was demonstrated in *C. difficile* isolates obtained from horses [S. S. Jang, L. M. Hansen, J. E. Breher, D. A. Riley, K. G. Magdesian, J. E. Madigan, Y. J. Tang, J. Silva, Jr., and D. C. Hirsh, abstract from the 35th Annual Meeting of Infectious Diseases Society of America 1997, Clin. Infect. Dis. **25**(Suppl. 2)**:**S266–S267, 1997], and Wong et al. (49) later reported on a clinical isolate of *C. difficile*, from among 100 isolates studied, for which the metronidazole MIC was >64 μg/ml. In our study, the highest rate of metronidazole resistance was observed in HIV-infected patients. This fact may be explained, among other reasons, by a theoretically higher probability of exposure to the antimicrobial agent on previous occasions.

To our knowledge, isolates of *C. difficile* that were not susceptible to vancomycin were reported only three times, from Poland in 1991 (16), although the study was performed by a disk diffusion method. Our data also showed that 13 strains (3.1%) had decreased susceptibility to vancomycin.

The first isolates of *C. difficile* not susceptible to vancomycin appeared in our institution in 1996, and since then we have had a low but persistent number of resistant isolates every year. This should be another reason to relegate vancomycin to a second position as the drug of choice for the treatment of CDAD. The risk of development of colonization with VRE and VRE superinfections in patients treated with oral vancomycin is already known (39, 40, 42).

The present paper demonstrates that this situation is far from anecdotal in our institution and that this problem was endemic during the study period. It could be argued that the situation in our institution may represent the epidemic spread of a single clone of a resistant strain, but molecular studies involving isolates from our institution have already shown that this is not the case (3). The typing study performed with some of the isolates as part of the present study demonstrates that a high degree of clonal heterogeneity was present. This fact was even observed with primer T7, described as an oligonucleotide with a low discriminatory ability (46), which is useful for the establishment of groups with high degrees of similarity for further analysis. Primer RAPD-#3 was able to show a high degree of clonal heterogeneity with a greater discriminatory ability.

It is also worth discussing at this point the fact that MIC breakpoints are usually set for isolates causing systemic infections and that they are based upon antimicrobial levels in blood. In this case, levels in the colon seem to be much more interesting than the levels in the circulation. For metronidazole and vancomycin, levels in the colon might even be high enough to kill "resistant" strains, although this issue has not been explored.

Our study tries to draw attention to a new problem in the emergence of antimicrobial resistance. Microbiology laboratories should not continue considering *C. difficile* a pathogen that cannot develop resistance to metronidazole and/or vancomycin. The immediate challenges in this area, in our opinion, are to demonstrate the extension of the problem in other institutions, to outline the predisposing factors for CDAD caused by strains which are not susceptible to vancomycin and/or metronidazole, and to clarify the impact of nonsusceptible strains on clinical therapy and their potential role in relapses. It is also necessary to demonstrate the mechanism of resistance and to look for new alternatives for the treatment and prevention of CDAD.

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